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EXPERIMENTAL ARTICLES

Biologically Active Lipids in Fungi of the *Pilobolaceae* Family

Ya. E. Sergeeva*, I. V. Konova*,[1], L. A. Galanina*, A. B. Gagarina, and N. M. Evteeva****

**Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*
***Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, ul. Kosygina 4, Moscow, 117977 Russia*

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Abstract—The synthesis of bioactive lipids in three species of lower mycelial fungi of the genus *Pilaira*, the family *Pilobolaceae*, has been studied. The pigmentation of these fungi was found to be determined by the presence of β-carotene in amounts of 14.8 (*P. moreaui*), 115.7 (*P. caucasica*), and 312.9 (*P. anomala*) μg/g. The fatty acid profiles of the fungi are distinguished by the presence of up to 50% essential fatty acids, which is typical of zygomycetes of the order *Mucorales.* The fungi grown in submerged cultures showed a correlation of the degree of unsaturation of total fatty acids and the contents of β-carotene.

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Key words: bioactive lipids, fungi, the family *Pilobolaceae.*

It is known that lower mycelial fungi of the order *Mucorales* synthesize essential fatty acids, β-carotene, and other carotenoids possessing pharmacologically important biological activity. Most of the carotenoid-producing fungi belong to the genus *Mucor* of the family *Mucoraceae*. The presence of yellow–orange pigments in the sporangiophores, sporangia, and spores of fungi from the genera *Mucor* and *Pilobolus* was reported as long ago as 1890. Later, these pigments and those of certain other genera of the family *Mucoraceae* were identified as carotenoids [1–4].

Some fungi, such as the mucor fungus *Blakeslea trispora* from the family *Choanephoraceae*, are known as active producers of β-carotene on an industrial scale [3, 5, 6]. The investigation of poorly studied taxa from the order *Mucorales* resulted in the discovery of new producers of the essential fatty acid C_{18} [7]. Certain fungi of the genus *Pilaira* of the family *Pilobolaceae* possess a yellow–orange pigmentation.

The aim of this work was to study the chemical nature of this pigmentation and to investigate the ability of various species of the genus *Pilaira* to synthesize biologically active lipids.

MATERIALS AND METHODS

Zygomycetes of the genus *Pilaira* used in this study (*P. anomala, P. moreaui*, and *P. caucasica*) were obtained from the All-Russia Collection of Microorganisms (VKM).

The fungi were cultivated in 250-ml flasks with 50 ml of a nutrient medium containing 4% glucose, 2% bactopeptone, 0.14% KH_2PO_4, 0.025% $MgSO_4 \cdot 7H_2O$, and 0.1% yeast extract in tap water (pH 6.2). The flasks were inoculated with an aqueous suspension of 6-day-old cells washed off the malt extract agar and incubated at 26–27°C on a shaker (220 rpm) for 6–7 days. Over this period, the culture was analyzed for pH, cell concentration, lipid content, composition and content of fatty acids, and content of pigments.

In order to determine the dry weight of biomass, a culture sample was filtered in a Buchner funnel, and the biomass was dried at 95°C to a constant weight.

Pigments were extracted from wet biomass with acetone. Extraction was continued until the biomass became colorless. The extracted pigments were transferred to hexane, dehydrated by adding anhydrous sodium sulfate, and concentrated in a rotary evaporator [8].

The pigments were qualitatively analyzed by thin-layer chromatography (TLC) on Silufol plates (Czech Republic) in a hexane–acetone (95 : 5, v/v) mixture [9] and also on Al_2O_3 plates in petroleum ether [10]. The markers were an authentic sample of synthetic β-carotene purchased from Hoffman LaRoche (Switzerland), crystalline microbial β-carotene (*Blakeslea trispora*, AO Uralbiopharm, Yekaterinburg, Russia), and carotenoids isolated from carrot. The concentration of

[1] Corresponding author; e-mail: inmi@inmi.host.ru

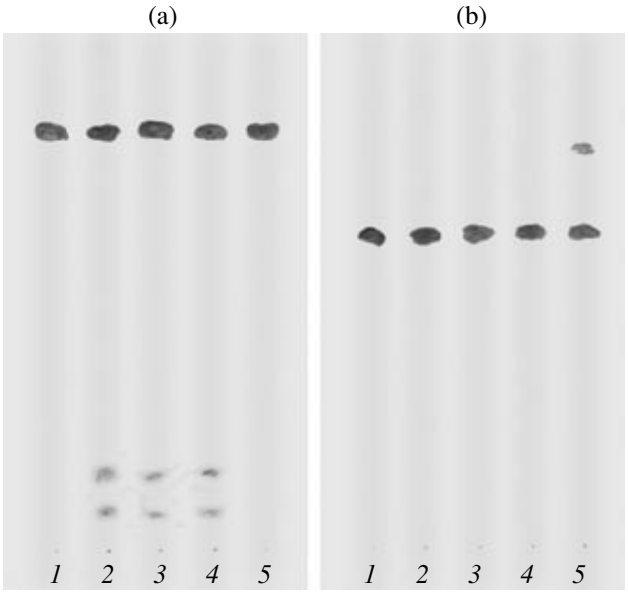

Fig. 1. TLC of extracted pigments on (a) Silufol plates in a hexane–acetone (95 : 5) mixture and (b) Al_2O_3 plates in petroleum ether: (*1*) synthetic β-carotene, (*2*) pigments extracted from *P. anomala,* (*3*) pigments extracted from *P. caucasica,* (*4*) pigments extracted from *P. moreaui,* and (*5*) β-carotene from *B. trispora* (panel a) and from carrot (panel b).

β-carotene in extracts was determined from their absorption spectra recorded with a Shimadzu UV–VIS–NIR scanning spectrophotometer (Japan). The content of β-carotene (in μg/g dry biomass) was calculated using the following formula:

$$\left(C = \frac{DVP}{E^{1\%}_{1\text{cm}} ml} 10^4\right), \quad (1)$$

where D is the absorbance of carotene at its spectral maximum (450 nm), V is the volume of hexane (ml), P is the degree of dilution of the original solution, $E^{1\%}_{1\text{cm}} = 2592$ is the extinction coefficient of β-carotene in hexane at 450 nm [11], m is the dry weight (in g) of the biomass used for pigment extraction, l is the optical path length of the cuvette (cm), and 10^4 is the conversion factor.

Fatty acids were analyzed as described by Kates [12]. Lipids were extracted with a chloroform–methanol (2 : 1, v/v) mixture and then with the same solvents but in another proportion (1 : 2). The extracted lipids had a pale yellowish color due to traces of pigments. The content of lipids was calculated with respect to the dry weight of the biomass.

Fatty acids were analyzed as methyl esters. Analysis was performed on a gas–liquid chromatograph, model 3700, equipped with a (1 m × 3 mm ID) glass column packed with Chromosorb WAW-DMCS-HP (80–100 mesh) containing 17% diethyleneglycol succinate. The carrier gas was argon at a flow rate of 50 ml/min. The column and evaporator were kept at 180 and 250°C, respectively. Fatty acids were identified by comparing the retention times of their methyl esters with those of the authentic samples. The content of particular fatty acids was expressed as a percentage of the total.

All the experiments were carried out in triplicate. The tables and the figures present the results of typical experiments.

RESULTS AND DISCUSSION

The TLC analysis of the extracted pigments in hexane–acetone solvent system showed the presence of one major spot with $R_f = 0.80 \pm 0.03$ and two minor spots with $R_f = 0.08 \pm 0.02$ and 0.15 ± 0.02. The major spot was found to correspond to carotenoids, whereas the minor spots presumably corresponded to products of carotenoid conversion. According to Kirchner [10], α- and β-carotenes cannot be separated on Silufol plates in hexane–acetone. For this reason, the extracts were also analyzed by TLC on Al_2O_3 plates in petroleum ether. This analysis showed the presence of one spot with $R_f = 0.62 \pm 0.02$, which corresponded to β-carotene. At the same time, the analysis of carrot extract on Al_2O_3 plates showed the presence of two spots, with $R_f = 0.61 \pm 0.03$ and 0.74 ± 0.02, which corresponded to α- and β-carotenes [10]. These data suggest that the fungi under study synthesize β-carotene. The typical chromatograms of carotenoid pigments of different origin are shown in Fig. 1.

A comparison of the absorption spectra of the hexane extracts and the authentic samples of β-carotene showed their identity (Fig. 2). This fact, together with the data of TLC (Fig. 1), confirms that the pigmentation of *P. anomala, P. moreaui*, and *P. caucasica* is due to the presence of β-carotene in these fungal species.

Konova *et al.* showed that fungi of the family *Pilobolaceae* also synthesize essential fatty acids, linoleic (*cis*-9,12-octadecadienoic) and γ-linolenic (*cis*-6,9,12-octadecatrienoic) [7]. The results of our studies of the cellular content of lipids, β-carotene, and essential fatty acids during the growth of the fungi are presented in Tables 1–4 and Fig. 3.

In the trophophase, the fungus *P. anomala* showed active growth during the first two days of cultivation, after which the biomass reached 61.4% of the maximum value (Fig. 3). During the next two days of growth, the increment of the biomass came to approximately 30%. Further increase in the biomass was small, if any. An active accumulation of cellular lipids took place when growth was not rapid. Specifically, during the third and fourth days of growth, the cellular content of lipids increased more than twofold (from 9.9 to 20.1%). This circumstance indicated that lipids accumulated more actively during transition to the stationary growth phase (in the idiophase), showing a common trend relative to the growth phases of fungi. It

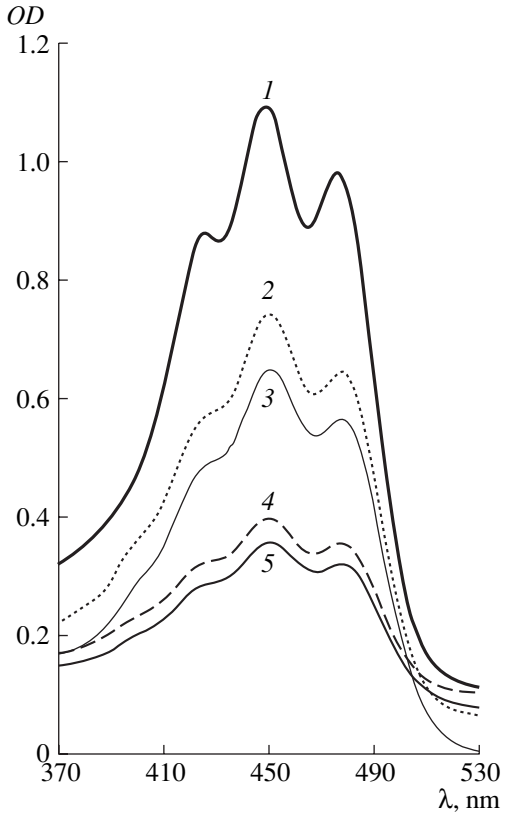

Fig. 2. Absorption spectra in hexane of (*1*) synthetic β-carotene, (*2*) β-carotene from *B. trispora,* (*3*) pigment extracted from *P. anomala,* (*4*) pigment extracted from *P. caucasica,* and (*5*) pigment extracted from *P. moreaui.*

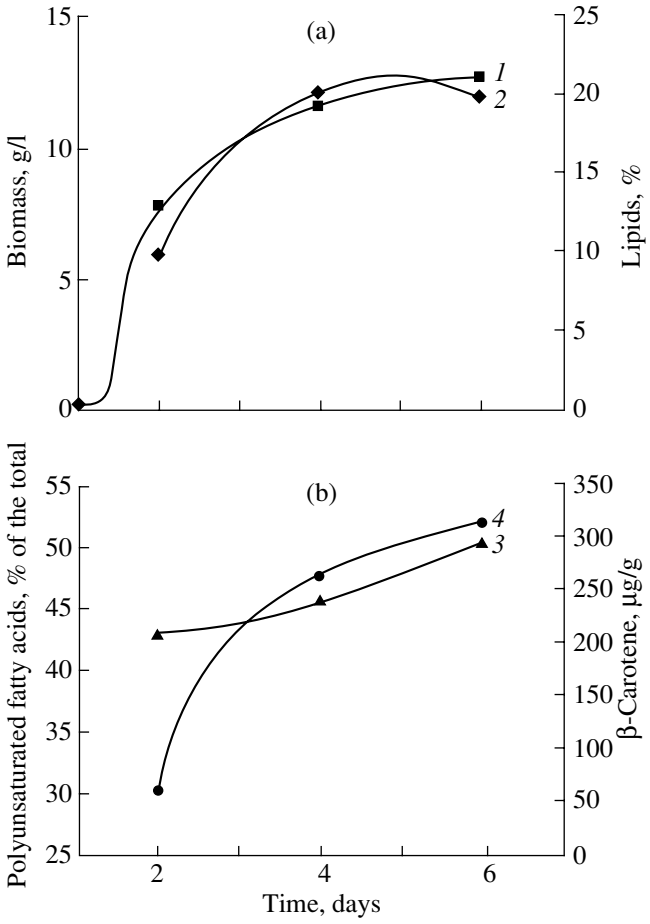

Fig. 3. Dynamics of (*1*) the biomass, (*2*) the lipid content, (*3*) the relative content of polyunsaturated fatty acids (% of total), and (*4*) the β-carotene content during the growth of *P. anomala.*

should be noted that β-carotene was most actively synthesized in the idiophase as well. Indeed, the content of β-carotene in the biomass comprised 62.0 μg/g (approximately 20%) after the first two days of growth and 263.9 μg/g after four days of growth. During the next two days of growth, when lipid content was constant, the cellular content of β-carotene increased by 20% and reached 312.9 μg/g.

The two other fungi under study showed similar dynamics of their cellular lipids and fatty acids relative to the growth phases (Tables 2 and 3), although the growth parameters of these fungi were different. In their β-carotene-producing capacity, the fungi ranked in the following order: *P. anomala* (312.9 μg/g after 6–7 days of growth), *P. caucasica* (115.7 μg/g), and *P. moreaui* (14.8 μg/g). For comparison, the content of β-carotene in its common source, carrot, comprises 80–110 μg/g [13].

The fatty acid profiles of the three fungi (Tables 1–3) were typical of the order *Mucorales.* Namely, their fatty acids ranged from C_{12} to C_{20} in length. The relative content of unsaturated fatty acids comprised up to 78.9% of the total, the major unsaturated acids being oleic ($C_{18:1}$), linoleic ($C_{18:2}$), and linolenic ($C_{18:3}$). The content of the two polyunsaturated essential fatty acids reached 50%.

The saturated fatty acids of the fungi could arbitrarily be divided into two groups: relatively short chain (the number of carbon atoms from 12 to 16) and relatively long chain (the number of carbon atoms from 18 to 20). The first group was represented by palmitic $C_{16:0}$ (11.9–23.9%), myristic $C_{14:0}$ (2.1–8.5%) and lauric $C_{12:0}$ (0.2–0.9%) fatty acids. The second group was mainly represented by stearic ($C_{18:0}$) acid (11.8–15.8% in *P. moreaui* and 4.1–9.6% in *P. anomala* and *P. caucasica*) and eicosanoic ($C_{20:0}$) acid (0.4–1.4%). The content of the minor unsaturated fatty acids (palmitoleic $C_{16:1}$ and eicosenoic $C_{20:1}$ fatty acids) varied from 0.6 to 2.7% in particular fungi.

A comparison of the unsaturation index of fatty acids [14] and the content of polyunsaturated fatty acids and β-carotene in the course of fungal growth (Table 4) showed a correlation between the syntheses of these compounds. At can be assumed that endogenous β-carotene may increase the level of polyunsaturated fatty acids due to its antioxidant effect on these acids.

Table 1. The relative content of fatty acids (% of the total) and their certain groups in the fungus *P. anomala*

Fatty acids	Cultivation time, h		
	48	96	144
$C_{12:0}$	0.2	0.2	0.3
$C_{14:0}$	2.1	3.8	4.3
$C_{15:0}$	Traces	0.2	0.3
$C_{16:0}$	11.9	14.2	13.5
$C_{16:1}$	1.0	1.5	1.7
C_x	Traces	Traces	Traces
$C_{18:0}$	5.7	4.5	4.1
$C_{18:1}$	33.4	27.4	24.0
$C_{18:2}$	21.9	29.4	31.0
$C_{18:3}$	20.9	16.2	19.2
$C_{20:0}$	1.3	1.4	0.9
$C_{20:1}$	1.6	1.2	0.8
Saturated short chain C_{12}–C_{16} acids	14.1	18.5	18.4
Saturated long chain C_{18}–C_{20} acids	7.0	5.9	5.0
Unsaturated C_{16}–C_{20} acids	78.9	75.6	76.6
Unsaturated C_{18} acids	76.3	72.9	74.2
Essential fatty acids	42.8	45.6	50.2

Table 2. Parameters of growth and lipogenesis in the fungus *P. moreaui*

Parameter	Cultivation time, h		
	48	96	144
Biomass, g/l	5.7	12.2	11.2
Lipids, %	9.4	11.9	13.4
Fatty acids, % of the total			
$C_{12:0}$	0.5	0.8	0.5
$C_{14:0}$	4.9	5.6	3.2
$C_{15:0}$	1.4	0.0	0.0
$C_{16:0}$	23.9	18.7	19.7
$C_{16:1}$	2.4	0.8	1.1
C_x	1.4	0.2	Traces
$C_{18:0}$	11.8	15.6	15.8
$C_{18:1}$	24.4	24.4	20.2
$C_{18:2}$	14.1	19.1	21.9
$C_{18:3}$	15.4	14.2	16.2
$C_{20:0}$	Traces	0.4	0.9
$C_{20:1}$	Traces	0.3	0.6
saturated short chain C_{12}–C_{16} acids	30.7	25.1	23.4
saturated long chain C_{18}–C_{20} acids	11.8	16.0	16.7
unsaturated C_{16}–C_{20} acids	56.2	58.7	59.9
unsaturated C_{18} acids	53.8	57.7	58.2
essential fatty acids	29.4	33.3	38.1

Earlier, Goncharova and Konova observed a correlation between the unsaturation index of lipids and the intensity of carotene synthesis at high concentrations of exogenous phosphate [16]. The phosphate concentration was found to influence the ultrastructural organization and the biochemical activity of the fungus *B. trispora* [15, 16].

Thus, along with the fungi of the families *Choanephoraceae* and *Mucoraceae,* the fungi of the family

Table 3. Parameters of growth and lipogenesis in the fungus *P. caucasica*

Parameter	Cultivation time, h	
	72	168
Biomass, g/l	13.2	13.9
Lipids, %	12.2	12.7
Fatty acids, % of the total		
$C_{12:0}$	Traces	Traces
$C_{14:0}$	8.5	5.7
$C_{15:0}$	Traces	Traces
$C_{16:0}$	18.1	20.2
$C_{16:1}$	2.0	2.7
C_x	Traces	Traces
$C_{18:0}$	7.9	9.6
$C_{18:1}$	21.7	15.7
$C_{18:2}$	26.0	28.8
$C_{18:3}$	14.7	15.8
$C_{20:0}$	1.0	1.4
$C_{20:1}$	Traces	Traces
saturated short chain C_{12}–C_{16} acids	26.6	25.9
saturated long chain C_{18}–C_{20} acids	8.9	11.0
unsaturated C_{16}–C_{20} acids	64.5	63.1
unsaturated C_{18} acids	62.5	60.4
essential fatty acids	40.7	44.7

Table 4. Correlation between the unsaturation index of lipids and the content of β-carotene in the fungi *P. anomala* and *P. caucasica*

Fungus	β-Carotene content, μg/g	Fatty acids	
		Essential acids, mg/g	Unsaturation index, Δ/mol*
P. anomala	312.9	74.6	1.46
P. caucasica	115.7	42.7	1.24

* Unsaturation index (Δ/mol) = (1 × % of monoenoic acids + 2 × % of dienoic acids + 3 × % of trienoic acids)/100 [14].

Pilobolaceae are promising for synthesis of pharmacologically important biologically active lipids.

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